

Induction of CTLs by DCs pulsed with K-ras mutant peptide on the surface of nanoparticles in the treatment of pancreatic cancer

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Abstract. The aim of this study was to investigate the role of specific cytotoxic T lymphocytes (CTLs) activated by dendritic cells (DCs) presenting cationic nanoparticles with the K-ras (12-Val) mutant peptide in the killing of different pancreatic cancer cell lines *in vivo* and *in vitro*. Peripheral blood DCs were induced by rhGM-CSF and IL-4 and cultured. DCs were sensitized by whole antigen of PANC-1 with expression of K-ras mutant, K-ras mutant peptide (K-ras+peptide) and cationic nanoparticles with K-ras mutant peptide (K-ras+peptide-CNP), respectively. Cell surface markers were measured by flow cytometry. Lymphocyte proliferation was detected by the ^3H -TdR test, and IL-12 and IFN- γ secretion was detected by ELISA. ^{125}I -UdR was used to measure the killing effect of CTLs. The antitumor activity of CTLs in tumor-bearing nude mouse models prepared with PANC-1 and SW1990 cells was evaluated. Results showed that, compared with K-ras+peptide, low concentrations of K-ras+peptide-CNP were effectively presented by DCs ($P<0.05$). CTLs induced by DCs pulsed with whole tumor antigen had a significantly greater killing effect ($P<0.05$) on PANC-1 and SW1990 pancreatic cancer cells compared with K-ras+peptide- and K-ras+peptide-CNP-induced CTLs. CTLs induced by DCs pulsed with K-ras+peptide and K-ras+peptide-CNP had a specific killing effect ($P<0.05$) on PANC-1 cells and no effect ($P>0.05$) on SW1990 cells. In conclusion, cationic nanoparticles with the K-ras (12-Val) mutant peptide can be

effectively presented by DCs at a low concentration. CTLs induced by K-ras+peptide-CNP had specific killing activity for the pancreatic cancer cell line with the K-ras mutant and significantly inhibited tumor growth and increased the survival time of tumor-bearing nude mice. Although this study confirmed that whole cell antigen induced a good antitumor immune response, the possibility of immune tolerance and autoimmunity which has been previously proven contribute to the difficulty in the application of this DC vaccine.

Introduction

Pancreatic cancer accounts for 1-5% of all malignant tumors (1). Its incidence has shown a clear upward trend in recent years with 230,000 new cases of pancreatic cancer each year worldwide (2). Pancreatic cancer lacks specific clinical manifestations. The surgical resection rate of pancreatic cancer is less than 20%, and it is not sensitive to radiotherapy or chemotherapy. The 5-year survival rate is less than 5%. The prognosis of pancreatic cancer has not improved in the last 20 years (3,4). Immunotherapy for pancreatic cancer mainly includes specific active immunotherapy, monoclonal antibody-directed therapy, cytokine therapy and adoptive cellular immunotherapy (5). Dendritic cells (DCs) are known to be the most powerful antigen presenting cells (APCs) in the human body (6,7). Studies have found that the K-ras gene mutation rate in pancreatic cancer patients was approximately 90% and that the gene mutation site was the ideal target for DC immunotherapy. In this study, we investigated the effects of DCs sensitized with different antigens and the killing effect of induced specific CTLs on different pancreatic cancer cell lines *in vivo* and *in vitro*.

Materials and methods

Materials. PANC-1 and SW1990 cell lines were purchased from ATCC. The cationic polymer β (1,4)-2-amino-2-deoxy-D-glucan was purchased from Sigma (USA). Human recombinant GM-CSF and IL-4 were purchased from R&D Systems (USA), and mouse anti-human CD80-PE, CD83-PE, CD86-PE, CD40-FITC, CD1a-FITC monoclonal antibodies were obtained from Immunotech (France). Ficoll (lymphocyte separation medium) was purchased from Second Plant of

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Abbreviations: CTLs, cytotoxic T lymphocytes; DCs, dendritic cells; mDC, mature dendritic cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; CNP, cationic nanoparticles; APCs, antigen-presenting cells; SI, stimulating index; TNF- α , tumor necrosis factor; PBS, phosphate-buffered saline; PBMCs, peripheral mononuclear cells; IFN- γ , interferon- γ ; FCS, fetal calf serum; ELISA, enzyme-linked immunosorbent assay

Key words: pancreatic cancer, dendritic cells, K-ras peptide, antigen, cationic nanoparticles, cytotoxic T lymphocytes, mutation

Shanghai Biochemical Reagents, and FCS was from Hyclone (USA). Iscove's modified Dulbecco's medium (IMDM) was from Gibco (USA). ^3H -TdR and ^{125}I -UdR were purchased from the Institute of Atomic Energy, China, and the flow cytometer was from BD Biosciences. The 550 microplate reader was from Bio-Rad. IL-12 and IFN- γ ELISA kits were purchased from Jingmei Co., Shenzhen, and the K-ras (12-Val) mutant peptide antibody was from Bayer Co. The K-ras mutant peptide KLVVVGAVGVGKSALTC (8) was synthesized by Saibaisheng Genentech, Beijing. BALB/c nude mice (5-8 weeks old, female) were purchased from the Shanghai Animal Center of Chinese Academy of Sciences and housed in a specific pathogen-free (SPF) environment.

Preparation of antigen and cell culture. The pancreatic cancer cell lines PANC-1 (with point mutations of K-ras, K-ras⁺) (9) and SW1990 (without point mutations of K-ras, K-ras⁻) were cultured in IMDM medium with 10% FCS at 37°C under 5% CO₂. After culturing for 72 h, passaging was performed. One week later, the cells were digested with trypsin containing 2% EDTA and washed twice with PBS. The supernatant was discarded, and the cells were counted. The concentration of PANC-1 cells was adjusted to $2 \times 10^7/\text{ml}$. The cells were frozen and thawed three times in liquid nitrogen and at 37°C, respectively. Centrifugation for 5 min at 100,000 rpm and filter sterilization were performed to prepare whole tumor cell antigen. Cationic polymer β (1,4)-2-amino-2-deoxy-D-glucan 0.2% (w/v) was dissolved in sodium acetate (pH 5.0). Then, K-ras epitope peptide KLVVVGAVGVGKSALTC was added, and the mixture was heated to 50-55°C. The liquid was mixed by magnetic force stirring, and cationic nanoparticles with the K-ras mutant peptide were prepared.

Separation of mononuclear cells. Fasting venous blood (20 ml) from a normal donor was collected under sterile conditions (the same sample was used in repeated experiments) and diluted with PBS at a 1:1 ratio. Mononuclear cells were separated from the lymphocyte separation medium (10), and a cell suspension was produced. A final concentration of 2×10^6 cells/ml was incubated for 2 h at 37°C under 5% CO₂ in an incubator. Then adherent cells were washed three times with IMDM. Non-adherent cells were washed away and collected for freezing. Adherent cells were scraped and used as monocytes.

Induction of imDC, mature (m)DC and antigen loading. Monocytes were cultured with cytokines (1,000 U/ml rhGM-CSF, 500 U/ml rhIL-4) (11), and half concentrations of cytokines were added every other day. On the fifth day, the following antigens were added: i) whole tumor cell antigen with tumor cells and DCs at a ratio of 1:10; ii) K-ras mutant antigen peptide (K-ras+peptide) (2.5, 5.0, 10.0 and 20.0 $\mu\text{g}/\text{ml}$); iii) cationic nanoparticles (CNP) with K-ras mutant antigen peptide (K-ras+peptide-CNP) (2.5, 5.0, 10.0 and 20.0 $\mu\text{g}/\text{ml}$); and iv) culture medium as control. The cells were cultured at 37°C in 5% CO₂. The medium was changed after 2 days, and TNF- α (10 $\mu\text{g}/\text{ml}$) was added for 48 h. The cell supernatant was collected.

Morphological observation of DCs pulsed with K-ras+peptide-CNP. The morphology of DCs was observed under scanning

and transmission electron microscopy after being sensitized by K-ras+peptide-CNP.

Detection of surface molecules of DCs. DCs induced by the different antigens were collected and centrifuged at 1500 rpm for 10 min. The cells were washed three times with PBS, incubated at 37°C for 30 min, and washed with PBS once more. FITC-rat anti-human CD80, CD83, CD86, CD1a, HLA-DR and K-ras (12-Val) mutant peptide antibodies were added. Fluorescent-labeled isotype Ig was used as the control. Flow cytometry was used for detection.

Detection of T lymphocyte proliferation. Lymphocytes were obtained from the same blood donors (separation method as described above). A total of $2 \times 10^5/\text{well}$ lymphocytes and $2 \times 10^4/\text{well}$ DCs loaded with the different antigens was mixed in 96-well plates and cultured at 37°C under 5% CO₂ for 5 days. ^3H -TdR (37 kBq/hole) was added for 12 h. Cells were collected, and the cpm value was measured with a liquid scintillation counter. The stimulating index (SI) was calculated: $\text{SI} = (\text{experimental cpm} - \text{background cpm})/(\text{control cpm} - \text{background cpm})$.

Detection of CTL killing effect. The lymphocytes from the proliferation assay (1×10^7) were used as effector cells. A total of 1×10^6 tumor cells in the logarithmic growth phase were cultured with 5 μCi ^{125}I -UdR and 5 mol 5-fluorouracil. Complete culture medium was added to a final volume of 1 ml, and the cells were cultured in an incubator for 2 h at 37°C in 5% CO₂. The cells were washed three times with IMDM to remove unlabeled ^{125}I -UdR. A γ -counter was used to determine the cell count. Cells with an average labeling rate >1 cpm were used as target cells. The target cells were adjusted to $5 \times 10^5/\text{ml}$ with IMDM culture medium containing 10% FCS. Effector and target cells were mixed at different ratios (1:6.25; 1:12.5; 1:25; 1:50), and complete culture medium was added for a final volume of 1 ml (set of 3 double tubes). A control group of target cells was used to determine the spontaneous release rate. The medium was centrifuged at 1,000 rpm for 3 min and cultured for 12 h at 37°C in 5% CO₂. After centrifugation at 2,000 rpm for 5 min, the cpm value was measured. Cytotoxic activity was expressed as the ^{125}I -UdR release percentage: ^{125}I -UdR release% = $(\text{cpm value of experimental group} - \text{cpm value of spontaneous release})/(\text{maximum release value of cpm} - \text{cpm value of spontaneous release}) \times 100\%$.

Animal experiments

Establishment of a tumor-bearing mouse model. PANC-1 and SW1990 cells in the logarithmic growth phase were suspended at a concentration of 1×10^7 cells/ml. Then, 0.2 ml of the cell suspension was inoculated subcutaneously into each BALB/c-nude mouse, and the survival time of the nude mice was observed. Ten days after inoculation, the mice were divided randomly into 5 groups with 10 mice in each group: i) whole cell antigen group which were injected with whole tumor antigen-induced CTLs, ii) K-ras+peptide-CNP group which was injected with K-ras+peptide-CNP-induced CTLs, iii) K-ras+peptide group which was injected with K-ras+peptide-induced CTLs, and iv) the control group injected with normal saline. Subsequently, 2×10^6 of the various antigen-induced CTLs were

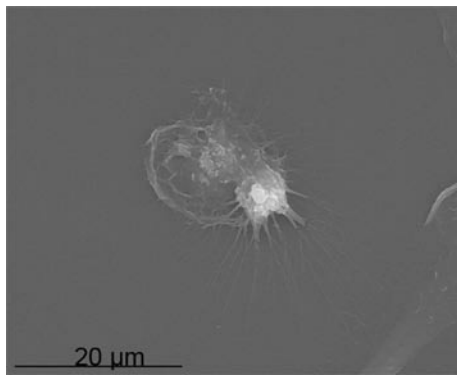


Figure 1. Morphological alterations of DCs on Day 7 (post-loading) of culturing under scanning electron microscopy. The cells exhibited a very irregular shape, and the processes varied widely, being either dendritic or petal-shaped.

injected intraperitoneally every other day for 10 consecutive days. Before injection, the long (L) and short (S) diameters of the tumors were measured. The tumor size was estimated by $V = (L \times S^2)/2$ (12). The survival time of the mice was monitored.

Statistical analysis. SPSS16.0 statistical software was used for statistical analysis. The measurement data were presented as mean \pm SD. The raw data underwent tests for homogeneity of variance before t-test and variance analysis with $P < 0.05$ as a statistically significant difference.

Results

Morphological observation. Four days after culturing, the DCs appeared in large clusters with a short thorn-like process at the surface of the cells. As time progressed, suspended cells gradually increased, and the thorn-like process increased, showing a flocculus-like shape characteristic of a typical dendritic cell.

DCs on Day 7 (post-loading) of culturing were observed under scanning electron microscopy. The cells exhibited a very irregular shape, and the processes varied widely, being either dendritic or petal-shaped (Fig. 1). Under transmission electron microscopy, after loading, dendritic or petal-shaped processes increased significantly with a clear nucleus, rich

organelles, numerous mitochondria, rich rough endoplasmic reticulum and rare lysosomes (Fig. 2).

Expression of cell surface markers by DCs loaded with different antigens. The expression levels of cell surface markers, CD1a, CD80, CD83 and HLA-DR, by DCs pulsed with whole tumor antigen were significantly higher than those of mature DCs ($P < 0.001$). Cell surface marker expression by DCs pulsed with K-ras+peptide and K-ras+peptide-CNP was not significantly different ($P > 0.05$). CD1a, CD80, CD83 and HLA-DR expression in DCs pulsed with whole tumor antigen was significantly higher than that in the K-ras+peptide and K-ras+peptide-CNP groups ($P < 0.05$). CD1a, CD80, CD83 and HLA-DR expression by DCs loaded with K-ras+peptide and K-ras+peptide-CNP was significantly higher than mDCs ($P < 0.05$), and CD86 expression between the groups showed no significant difference ($P > 0.05$) (Table I).

Detection of the K-ras mutant antigen peptide presented by DCs. The antigen presenting capability of DCs was measured by flow cytometry using the K-ras mutant monoclonal antibody (12-Val). After being treated with 5 $\mu\text{g/ml}$ K-ras+peptide-CNP, the antigen-presenting rate of DCs was 55.85%. No significant difference in the presenting capability of DCs was found when compared with the DCs treated with 10 $\mu\text{g/ml}$ K-ras+peptide-CNP (56.13%) ($P > 0.05$) (Fig. 3A). Similarly, the presenting capability of DCs was not significantly different when they were treated with high concentrations of antigens (10 and 20 $\mu\text{g/ml}$) ($P > 0.05$). However, at low concentrations (2.5 and 5 $\mu\text{g/ml}$), the antigen-presenting rate of DCs in the K-ras+peptide-CNP group was significantly higher than that of the K-ras+peptide group ($P < 0.05$) (Fig. 3B).

Effect of different antigens on the production of IL-12 by DCs. After treatment with low concentrations (2.5, 5 and 10 $\mu\text{g/ml}$) of K-ras+peptide and K-ras+peptide-CNP, the production of IL-12 by DCs in the K-ras+peptide-CNP group was significantly higher than that of the K-ras+peptide group ($P < 0.05$). The production of IL-12 by DCs showed no significant difference when treated with high concentrations of antigen ($P > 0.05$) (Fig. 4A). DCs loaded with different antigens had significant differences in IL-12 secretion levels ($P < 0.05$). DCs of the tumor cell antigen group produced the highest levels of

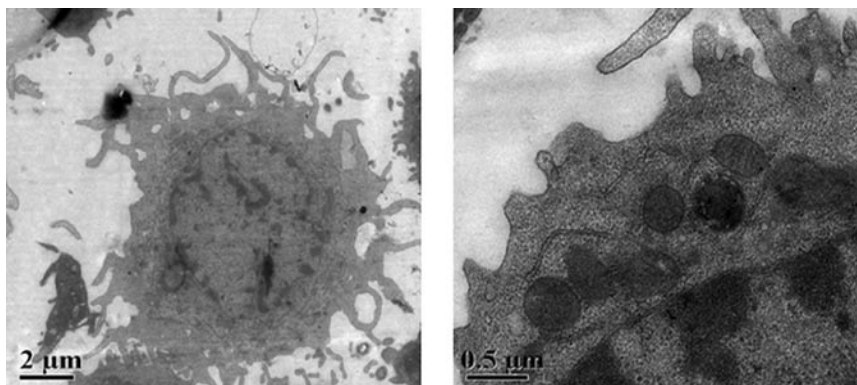


Figure 2. Morphological alterations of DCs on Day 7 (post-loading) of culturing under transmission electron microscopy. Dendritic or petal-shaped processes increased significantly with a clear nucleus, rich organelles, numerous mitochondria, rich rough endoplasmic reticulum and rare lysosomes.

Table I. Surface markers of DCs induced by different antigens (% positive cells, mean \pm SD, n=6).

| Groups | CD1a | CD80 | CD83 | CD86 | HLA-DR |
|--------------------------------------|----------------|----------------|----------------|----------------|----------------|
| mDC | 22.6 \pm 3.6 | 49.4 \pm 3.2 | 38.5 \pm 4.6 | 72.6 \pm 5.6 | 66.5 \pm 4.6 |
| DC (tumor, T:DC=1:10) | 55.2 \pm 4.5 | 82.6 \pm 4.9 | 76.3 \pm 5.1 | 80.9 \pm 6.7 | 93.2 \pm 5.8 |
| DC (K-ras+peptide, 10 μ g/ml) | 35.1 \pm 4.3 | 62.2 \pm 5.8 | 51.1 \pm 4.9 | 74.4 \pm 5.2 | 82.4 \pm 4.4 |
| DC (K-ras+peptide-CNP, 5 μ g/ml) | 38.4 \pm 3.7 | 67.5 \pm 4.7 | 58.4 \pm 6.3 | 75.3 \pm 4.8 | 86.1 \pm 5.3 |

DC (tumor, T:DC=1:10) vs. mDC, $P<0.001$ except CD86; DC (K-ras+peptide, 10 μ g/ml) and DC (K-ras+peptide-CNP, 5 μ g/ml) vs. mDC, $P<0.05$ except CD86; DC (tumor, T:DC=1:10) vs. DC (K-ras+peptide, 10 μ g/ml) and DC (K-ras+peptide-CNP, 5 μ g/ml), $P<0.05$ except CD86.

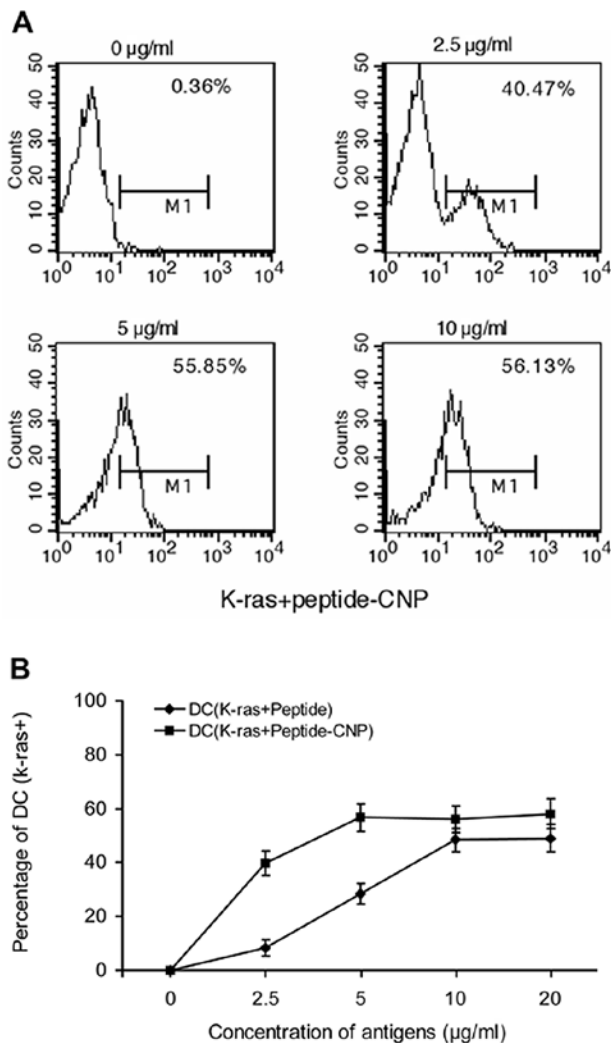


Figure 3. Effect of DC loading with different concentrations of K-ras+peptide and K-ras+peptide-CNP by flow cytometry. (A) After being treated with 5 μ g/ml K-ras+peptide-CNP, the antigen-presenting rate of DCs was 55.85%. No significant difference in the presenting capability of DCs was found compared with DCs treated with 10 μ g/ml K-ras+peptide-CNP (56.13%) ($P>0.05$). (B) Similarly, the presenting capability of DCs was not significantly different when they were treated with high concentrations of antigens (10 and 20 μ g/ml) ($P>0.05$). However, at low concentrations (2.5 and 5 μ g/ml), the antigen presenting rate of DCs in the K-ras+peptide-CNP group was significantly higher than that of the K-ras+peptide group ($P<0.05$).

IL-12, followed by the K-ras+peptide-CNP and K-ras+peptide groups, while mDCs secreted minimal IL-12 (Fig. 4B).

Effect of DCs loaded with different antigens on peripheral blood mononuclear cell (PBMC) proliferation and interferon (IFN)- γ production. PBMC proliferation and IFN- γ production induced by DCs loaded with whole tumor antigen were significantly greater than when DCs from the other groups were used ($P<0.01$). Those in the K-ras+peptide-CNP group were greater than those of the K-ras+peptide and mDC groups ($P<0.05$). The K-ras+peptide group was greater than the mDC group ($P<0.05$), as shown in Fig. 5.

Effect of DCs loaded with different antigens on the activity of tumor-specific CTLs induced in vitro. CTLs induced by DCs loaded with PANC-1 total antigen had the strongest killing effect on PANC-1 cells. Compared with the K-ras+peptide-CNP and K-ras+peptide groups, the P -value was <0.05 . Compared with the mDC group, the P -value was <0.01 . Among the CTLs induced by DCs loaded with K-ras+peptide-CNP and K-ras+peptide, no significant difference was noted in the killing effect on PANC-1 cells ($P>0.05$) but were significantly stronger than those of the mDC group ($P<0.05$). CTLs induced by DCs loaded with PANC-1 whole antigen had the strongest killing effect on SW1990 cells, which was significantly higher than in the other three groups ($P<0.05$). CTLs induced by DCs loaded with K-ras+peptide-CNP and K-ras+peptide did not have significantly different killing effects against SW1990 cells compared with the mDC group ($P>0.05$). These results indicated that CTLs induced by DCs loaded with the K-ras mutant epitope peptide were able to kill PANC-1 tumor cells with the K-ras mutant antigen but not SW1990 tumor cells without the K-ras mutant tumor antigen (Fig. 6).

Effect of CTLs induced by different tumor antigens on the survival time of tumor-bearing nude mice. A total of 2×10^6 CTLs induced by DCs loaded with different antigens were injected intraperitoneally, and the survival time of the nude mice was observed. The effects of the whole antigen group, K-ras+peptide-CNP group and K-ras+peptide group on the survival time of PANC-1 tumor-bearing mice were significantly different compared with the control group ($P<0.01$) (Fig. 7A). Prolongation of survival time in the whole antigen group was most significant and was significantly different compared with the other two groups ($P<0.05$). No significant difference was found between the K-ras+peptide-CNP group and the K-ras+peptide group ($P>0.05$). The effect of the whole antigen groups on the survival time of SW1990 tumor-bearing mice was significantly different compared with the K-ras+peptide-

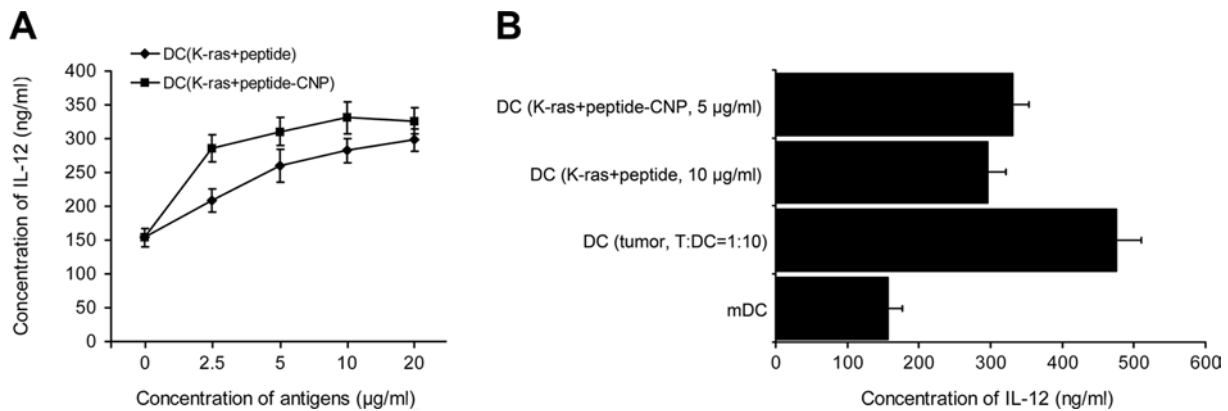


Figure 4. Effect of DC loading with different antigens on the production of IL-12. The production of IL-12 by DCs in the K-ras+peptide-CNP group was significantly higher than that of the K-ras+peptide group ($P < 0.05$), yet no significant difference was noted when treated with a high concentration (20 $\mu\text{g/ml}$) of the antigen ($P > 0.05$) (A). DCs of the tumor cell antigen group produced the highest levels of IL-12, followed by the K-ras+peptide-CNP and K-ras+peptide groups, while mDCs secreted minimal IL-12 (B).

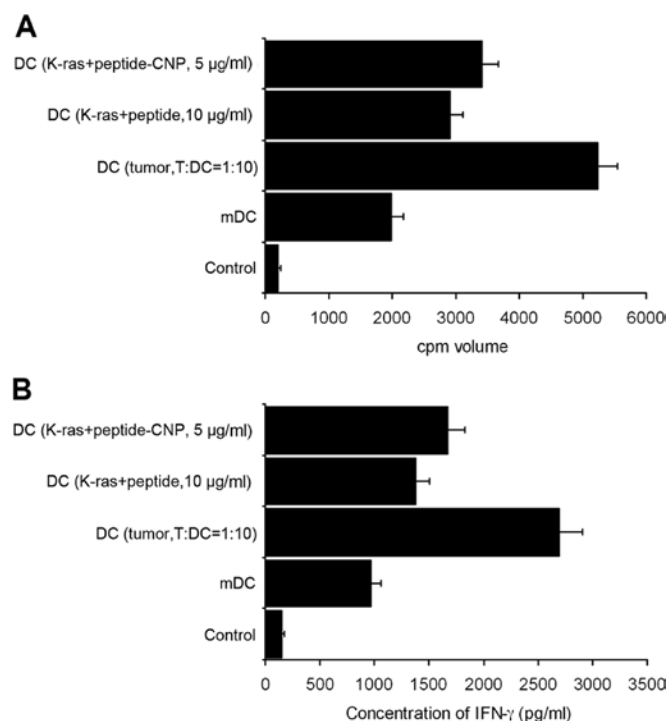


Figure 5. Effect of DC loading with different antigens on the (A) induction of proliferation and (B) IFN- γ production in PBMCs. PBMC proliferation and IFN- γ production induced by DCs loaded with whole tumor antigen were significant greater than when DCs from other groups were used ($P < 0.01$). These effects in the K-ras+peptide-CNP group were greater than those of the K-ras+peptide and mature DC groups ($P < 0.05$).

CNP group, K-ras+peptide group and control group ($P < 0.01$) (Fig. 7B). The mice had a prolonged survival time. There was no significant difference between the other groups ($P > 0.05$). Both results showed that the CTLs induced by the K-ras mutant epitope peptide significantly prolonged the survival time of PANC-1 tumor-bearing mice.

Discussion

Approximately 10% of pancreatic cancer is caused by genetic susceptibility (13), of which most are due to gene mutations, including K-ras, CDKN2A, BRCA2, DPC4, p53, AKT2 and

p16 (14-18). The K-ras gene is located on chromosome 12p12 and encodes the ras protein. The K-ras gene point mutation generally occurs in codons 12, 13 and 61, with a highest mutation rate at codon 12. Research has shown that the K-ras gene mutation rate in pancreatic cancer is approximately 90% (19). It has been found that a point mutation of codon 12 of the K-ras gene is an early event in pancreatic cancer (20). The clinical significance of the K-ras gene mutation for effective diagnosis of early pancreatic cancer may exceed imaging and cytology examination (21). Gene targeting and immunotherapy for codon 12 of the K-ras gene may aid in the diagnosis and treatment of pancreatic cancer (22).

Research in the biological treatment for tumors has confirmed that DC vaccines have significant value in the treatment of malignant tumors and infectious diseases. However, DCs require different forms of modification *in vitro* before the application of tumor therapy to activate and improve the quantity and quality of the DCs. These modifications include lysed tumor antigen, epitope peptides, RAN transfection, apoptosis of tumor cells, cytokines and heat shock proteins. At present, DCs are mainly derived from CD34⁺ hematopoietic stem cells or PBMCs (23).

In the present study, DCs were isolated and cultured from PBMCs using the traditional method. The typical morphology of DCs was shown under scanning and transmission electron microscopy. Immune analysis showed that after stimulation with different antigens (whole-cell antigen and the K-ras mutant antigen peptide), surface molecular markers of mature DCs appeared. Cation vector is a polymer material mainly used for enhancing gene transfection efficiency. We found that the cation vector combined with antigen peptide enhanced the phagocytosis of the antigen into the DCs and increased the antigen load of the DCs, which may be related to molecular size and release ability (24). The epitope can be presented at an early stage, but, with the increase in the epitope concentration, the presented epitopes at the surface of the DCs did not increase significantly, which may be due to the promotion of DC phagocytosis by the cationic carrier rather than the presenting function. Thus, when the loaded antigen was saturated, increasing the antigen did not cause an increase in epitopes. Research on the

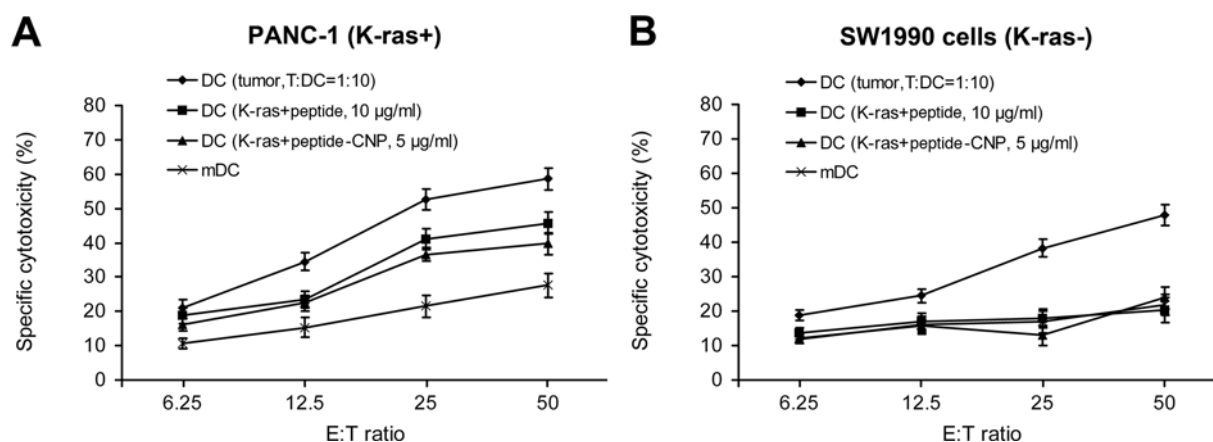


Figure 6. Effect of DC loading with different antigens on the induction of tumor-specific CTL cytotoxicity against (A) PANC-1 (K-ras⁺) and (B) SW1990 cells (K-ras⁻) *in vitro*. CTLs by DCs loaded with PANC-1 total antigen had the strongest killing effect on PANC-1 cells. Compared with the K-ras+peptide-CNP and K-ras+peptide groups, the P-value was <0.05. Compared with the mDC group, the P-value was <0.01. CTLs by DCs loaded with PANC-1 whole antigen had the strongest killing effect on SW1990 cells. CTLs by DCs loaded with K-ras+peptide-CNP, K-ras+peptide and mDC did not exhibit a significant killing effects against SW1990 cells.

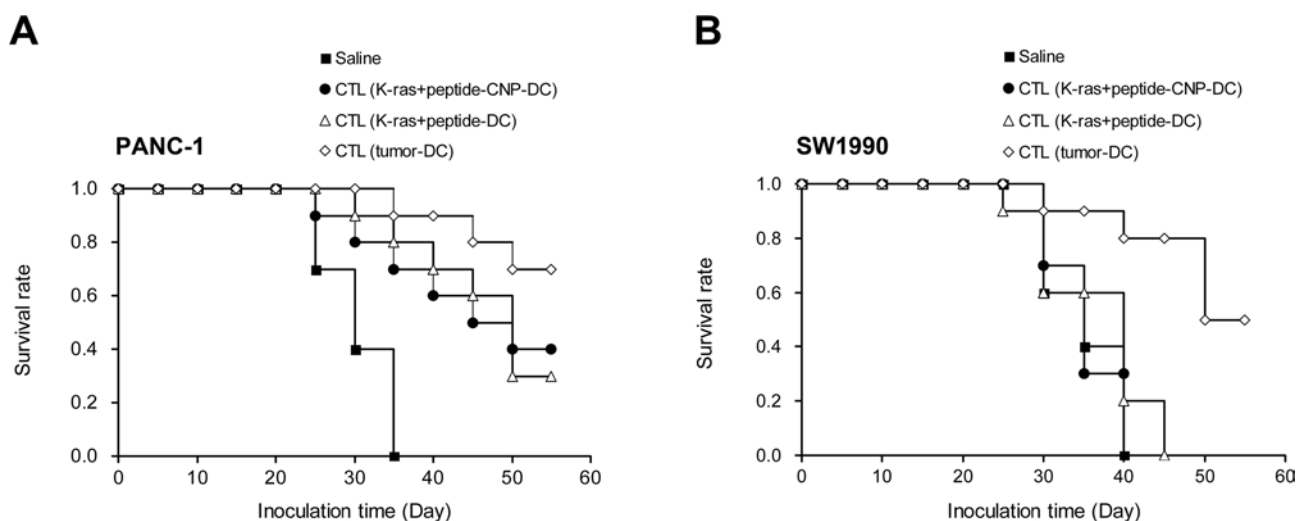


Figure 7. Survival of nude mice inoculated subcutaneously in the back with PANC-1 and SW1990 cells after immunotherapy with CTLs induced by DCs loaded with different antigens. Prolongation of survival time in the whole antigen group was most significant and was significantly different compared with the other two groups ($P < 0.05$) (A). The effect of the whole antigen groups on the survival time of SW1990 tumor-bearing mice was significantly different compared with the K-ras+peptide-CNP group, K-ras+peptide group and control group ($P < 0.01$) (B).

K-ras mutant peptide as a loading antigen has been reported. Pure peptide is easily degraded, which limits its application. A relatively low amount of peptide can saturate the presenting DCs, while a large amount of antigen can easily cause immune tolerance. A small amount of cationic nanoparticles with K-ras mutant antigen peptides can be phagocytized and presented at an early stage, which is very important in immune therapy for cancer. These nanoparticles may be a new method for loading DCs with an antigen, and the antigen is specific with less cross-reactivity. The advantages of this method are unparalleled. It can significantly promote the proliferation and cytotoxic activity of CTLs compared with CTLs induced by DCs modified with ordinary peptide, which may be related to the promotion of IL-12 secretion by DCs and increased immune activity (25,26). To confirm the specificity of the

immune response of CTLs induced by DCs pulsed with specific tumor antigen, PANC-1 cells with the K-ras gene mutation and SW1990 cells without the K-ras gene mutation were used (9). The results showed that CTLs induced by DCs sensitized by the K-ras antigen had a specific cytotoxic effect on PANC-1 cells but no significant inhibition of SW1990 cells ($P < 0.05$). Therefore, DC vaccines loaded with the K-ras point mutation antigen peptide are important in specific immunotherapy for pancreatic cancer patients with a ras gene mutation.

Of the three types of antigens, whole cell antigen had the greatest impact on the DCs. It promoted higher expression of surface mature molecules of DCs (CD80, CD83, CD86, HLA-DR, CD1a), which may be due to the complexity of its antigen components and a higher level of cancer antigen. In

the present study, we found that CTLs induced by DCs loaded with PANC-1 cell antigen demonstrated strong cytotoxic effects not only on PANC-1 cells but also on SW1990 cells. Many effective antigens in PANC-1 cells, including K-ras protein, were phagocytized and presented by DCs in this experiment; one or a variety of antigens was the effective recognition component of SW1990 cells by CTLs. Thus, DCs sensitized by PANC-1 cell antigen have a greater therapeutic effect on SW1990 pancreatic cancer cells *in vivo* and *in vitro*. Although this study confirmed that whole cell antigen induced an ideal antitumor immune response, it contained the normal cellular components, which make immune tolerance and autoimmunity possible. Additionally, the tumor antigen content cannot be easily controlled, which causes difficulty in the application of DC-sensitized antigen (27-29).

In the present study, whole tumor cell antigen-, K-ras+peptide- and K-ras+peptide-CNP-sensitized DCs promoted the expression of surface molecules of mature DCs and enhanced the cytotoxic activity of CTLs on target cells, IL-12 secretion by DCs and IFN- γ secretion by T cells. All CTLs induced by the three types of antigen were able to kill target cells effectively. K-ras+peptide-CNP- and K-ras+peptide-sensitized DCs exhibited more specificity for the target cells. Sensitization by K-ras+peptide-CNP was more effective than K-ras+peptide. *In vivo* experiments also support these results, which lay the foundations for the future development of DC vaccines for pancreatic cancer.

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